Identification and Characterization of a Low Phytic Acid Wheat

Mary Guttieri, David Bowen, John A. Dorsch, Victor Raboy, and Edward Souza*

ABSTRACT

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, or Ins P_6) is the most abundant storage form of P in seeds, yet indigestible by humans and nonruminant livestock. A wheat (*Triticum aestivum* L.) mutant is described herein with greatly reduced seed phytic acid P but little change in seed total P, similar to *lpa*1-type mutants described in other grain species. One nonlethal mutant from 562 ethyl-methanesulfonate (EMS) mutagenized M_2 lines was identified with a high inorganic phosphate (HIP) phenotype and designated Js-12-LPA. Js-12-LPA homozygotes produced seed in which phytic acid P represented 48.2% of seed total P, in contrast to 74.7% of seed total P in nonmutant or wild-type control, Js-12-WT. The inorganic portion of seed P was increased from 9.1% in Js-12-WT to 50.1% in Js-12-LPA, with little effect on total seed P. Weight distributions among milling fractions were similar for the Js-12-LPA and Js-12-WT genotypes. The low phytic acid trait altered the distribution of total P within the kernel, increasing the P content of the central endosperm and decreasing the P content of the bran. The low phytic acid trait decreased the phytic acid concentration in the bran by 43% and increased the inorganic P concentration in the bran nearly four-fold. Inheritance data of F_2 and $F_{4:6}$ families was inconsistent with a single-gene mutation and suggests the involvement of two or more genes. This low phytic acid wheat mutant is a genetic resource for studying the biology of seed phytic acid metabolism and wheat quality improvement.

MYO-INOSITOL-1,2,3,4,5,6-HEXA*KIS*PHOSPHATE is ubiquitous in eukaryotic cells, where it is typically the most abundant inositol phosphate (Ins P) (Sasakawa et al., 1995). First observed as an abundant P-containing compound in seeds, Ins P_6 in plant species is thus referred to as "phytic acid" in agronomic literature (Cosgrove, 1980). Applied interest in seed phytic acid is the result of its role in human and livestock nutritional quality, as well as P management in integrated agricultural production systems. According to Shears (2001) and Raboy (1997), Ins P_6 is a major pool in both P and Ins P metabolism. Phytic acid P typically represents from 65 to 85% of seed total P and >90% of free Ins polyphosphates (Raboy, 1997). Trace levels (<10% of total Ins P) of Ins tris-, tetra*kis*-, and penta*kis*phosphates (Ins P's with three, four, or five phosphomonoesters, respectively), as well as pyrophosphate-containing Ins P's more highly phosphorylated than Ins P_6, also are observed in mature, wild-type seeds (Dorsch et al., 2003).

In humans, diets high in phytate can significantly decrease the absorption of essential micronutrients such as Ca (Kies, 1985), Fe (Brune et al., 1992), and Zn (Sandström et al., 1987). Phytate forms chelates with these divalent minerals, which reduces bioavailability to humans (Jacobsen and Slotfeldt-Ellingsen, 1983). In developing countries, flatbreads may be prepared from whole-wheat flour or high (≈95%) extraction flour. The phytate in wheat grain is found predominantly in the aleurone layer, which remains attached to the pericarp during milling and therefore is concentrated in the bran fraction. Although phytate decreases in proportion to fermentation time, in chapati bread, the phytate concentration was only 18 to 24% lower than the concentration in the whole-wheat flour from which it was prepared (Anjum et al., 2002). The phytate concentration in chapati breads was six- to nine-fold higher than in breads prepared from straight-grade flour. However, mineral concentrations generally were significantly higher in whole-grain flour than in straight-grade flour, which might offset the effect of phytate on human nutrition. Human bioavailability studies will be necessary to confirm this hypothesis.

Nonlethal recessive mutations that decrease seed phytic acid concentration have been isolated in maize (*Zea mays* L.; Raboy and Gerbasi, 1996; Raboy et al., 2000), barley (*Hordeum vulgare* L.; Larson et al., 1998; Rasmussen and Hatzack, 1998), rice (*Oryza sativa* L.; Larson et al., 2000), and soybean [*Glycine max* (L.) Merr.; Hitz et al., 2002; Wilcox et al., 2000]. These *low phytic acid* (*lpa*) mutants affect the partitioning of P into phytic acid, inorganic phosphorus (P_i), and Ins P's with five or fewer P esters (Raboy, 2002). The *lpa* mutations are divided into two groups, designated *lpa*1 and *lpa*2. Phytic acid P reductions in *lpa*1 mutants are counterbalanced by molar-equivalent increases in P_i. In contrast, phytic acid P reductions in *lpa*2 mutants are counterbalanced by increases in both P_i and non-Ins P_6 Ins P's (Ins P's of lower phosphorylation). The isolation and characterization of seed P and Ins P phenotype of a heritable wheat *lpa*1-like mutant is described here.

MATERIALS AND METHODS

Mutant Isolation

Seed of the breeding line A95631S-Js-12 was mutagenized with 2% EMS. A95631S-Js-12 has the pedigree 'Kanto 79'/2*IDO488. IDO488 is a soft white spring wheat breeding line with the pedigree PI 294994/4*'Centennial'. M_1 plants were grown in the greenhouse. Approximately 50% of the M_1 seeds either did not germinate or senesced without setting seed. An M_2 row was planted in the field from each of 562 M_1 green-

M. Guttieri, D. Bowen, and E. Souza, Univ. of Idaho, Aberdeen Research and Extension Center, P.O. Box 870, Aberdeen, ID 83210; J. Dorsch, BASF Corp., Research Triangle Park, NC 27709; V. Raboy, USDA-ARS Small Grains and Potato Research Unit, P.O. Box 607, Aberdeen, ID 83210; J. Dorsch is a former postdoctoral fellow of the USDA-ARS. Research supported by the Idaho Agric. Exp. Stn. Project IDA1222, Manuscript no. 3725. Received 1 May 2003. *Corresponding author (esouza@uidaho.edu).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: AACC, American Association of Cereal Chemistry; EMS, ethyl-methanesulfonate; HIP, high inorganic phosphate; Ins P, inositol phosphate; Ins P_6, *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate; *lpa*, *low phytic acid*; P_i, inorganic phosphorus.

house-grown plants. A single spike was harvested from each of 10 plants in each of the 562 M_2 rows. Five M_3 seeds were sampled from each M_2 plant, individually crushed with a hammer blow or lab press, and incubated overnight at 4°C in 0.4 *M* HCl (10 μL per mg seed). The extracts were then briefly vortexed, and allowed to settle for a minimum of 0.5 h. Aliquots were assayed for P_i using the microtitre plate colorimetric assay as previously described (Larson et al., 2000; Raboy et al., 2000). Nonmutant wheat seeds typically contain ≤0.5 mg P_i g^{-1}. Seed with >1.0 mg P_i g^{-1} were considered HIP phenotypes.

One spike from a single M_2 plant (A95631S-Js-12-333Mu-4) was identified that appeared heterogeneous for the HIP phenotype. Remnant M_3 seed from this spike was planted in the greenhouse. Ten M_4 kernels from each M_3–derived plant were evaluated for HIP phenotype. Two homozygous M_3 plants were identified from the single M_2 plant. One M_3 plant (A95631S-Js-12-333Mu-4-6) was determined to be homozygous for the HIP phenotype, and another M_3 plant (A95631S-Js-12-333Mu-4-8) was determined to be homozygous for the wild-type inorganic P phenotype, on the basis of analyses of 10 M_4 kernels from each M_3 plant. Remnant M_4 kernels from these two plants, hereafter designated as Js-12-LPA and Js-12-WT, were planted in the field at Aberdeen, ID, in 2000. Individual M_4 plants were harvested and homozygosity of the HIP phenotype was confirmed by evaluations of single kernels.

Grain Production

Homozygous M_5 seed from the 2000 Aberdeen field trial was bulked and planted in El Centro, CA. M_6 seed harvested from California was planted in Tetonia, ID, in May 2001. Grain was produced with irrigation by overhead sprinklers using agronomic practices standard for the region. M_7 grain was harvested in September 2001.

Assay for High Inorganic Phosphate Phenotype

Kernels were individually crushed and extracted overnight in 10 μL 0.4 N HCl mg^{-1} (approximate seed weight). A 10-μL aliquot of each extract was then assayed for P_i in microtiter plates, using a modification of the colorimetric method of Chen et al. (1956). Phenotype was evaluated visually relative to P standards, as described by Larson et al. (2000).

Analyses of Seed Phosphorus Fractions

Analytical protocols for quantitative chemical compositions were as described (Larson et al., 2000; Raboy et al., 2000; Dorsch et al., 2003). Briefly, seed total P was determined colorimetrically (Chen et al., 1956) following wet-ashing of aliquots of tissue. Phytic acid P was determined by the ferric-precipitation method. Inorganic P was determined colorimetrically following extraction of tissue in 12.5% (w/v) 2,4,6-Trichloroanisole: 0.25 m*M* $MgCl_2$. The experiment was conducted as a completely randomized design with three replications. Analysis of variance was used to test the effect of genotype.

Anion-exchange HPLC analyses of seed Ins P's using post-column metal-dye detection were performed as described (Larson et al., 2000; Raboy et al., 2000; Dorsch et al., 2003). Seeds were extracted in 0.4 *M* HCl (10 v/w) and 200 μL of supernatant was diluted with double-distilled H_2O (to 1.0 mL) and passed through a 0.2-μm filter. An aliquot (200 μl) was then fractionated on a Dionex IonPac AS7 anion-exchange column, equipped with a Dionex IonPac AG7 guard column, which had been equilibrated with 10 m*M* methyl piperazine, pH 4.0 (Buffer A). The Ins P's were eluted with the following gradient system at a flow rate of 1.0 mL min^{-1}: initial condition, 100% Buffer A; 1 to 45 min, a linear gradient from 0 to 80% 0.5 *M* NaCl pH 4.0, in 10 m*M* methyl piperazine pH 4.0 (Buffer B); 45 to 60 min, 20 to 100% Buffer A. The column eluent was mixed with metal dye detection colorimetric reagent (0.015% $FeCl_3$:0.15% sulfosalicylic acid in 0.05 *M* methylpiperazene, pH 4.0) at a flow rate of 0.5 mL min^{-1}, using an Upchurch PEEK high pressure mixing tee (VWR Scientific, Philadelphia, PA) and an Eldex Model B-100-S metering pump (Eldex Laboratories, Inc., Menlo Park, CA), and the mixture passed through a 100-cm reaction coil before peak detection via absorbance at 550 nm. The Ins P in a sample peak was calculated using a standard curve obtained via the analysis of seven Ins P_6 standards prepared using commercially-obtained Na Ins P_6 (Sigma). These standards contained 25, 50, 75, 100, 125, 150, and 200 n*M* Na Ins P_6, which yielded area units ($\times 10^6$) of 5.9, 14.5, 22.0, 31.1, 39.1, 45.3, and 59.5, respectively.

Milling

Grain (1 kg) from the 2001 Tetonia production was tempered by the standard American Association of Cereal Chemistry (AACC; American Association of Cereal Chemists, 1995) Method 26-10 for soft wheat. Tempered grain was milled using a Brabender Quadrumat Senior Mill (C.W. Brabender Instruments, Inc., South Hackensack, NJ; AACC Method 26-21A). Flour protein concentration was determined with a near-infrared analyzer (Instalab 600, Dickey-John Corp., Auburn, IL, AACC method 39-10A), calibrated by automated combustion analysis of total N content (LECO Model NFP-428, LECO Corp., St. Joseph, MO), and corrected to 120 g kg^{-1} moisture.

Elemental Analysis

Mineral element composition of milling fractions was determined by the University of Idaho Analytical Services Laboratory using a Perkin-Elmer Optima 3200 ICP-OES (Inductively Coupled Plasma–Optical Emission Spectrometer) to quantify aqueous constituents following nitric acid digestion of the milling fractions. Mineral concentrations tested included Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, V, Zn, and Y. The experimental design was a randomized complete block with two replications. Analysis of variance was used to test the effect of genotype, milling fraction, and the interaction between genotype and milling fraction on concentration of each element.

Segregation Studies

Segregation of the HIP phenotype was evaluated in the cross Js-12-LPA/IDO563. IDO563 is an elite soft white spring breeding line of the pedigree 'Kanto 79'/2*IDO488 and is a sister selection to the line A95631S-Js-12 used for mutagenesis. The F_1 generation was grown in the greenhouse to produce F_2 seed. Eighty-six F_2 plants then were grown in the greenhouse. Six plants produced <20 kernels or produced kernels with an average weight of <10 mg and were excluded from the segregation analysis. These small kernels were highly shriveled and had not undergone normal seed development. The F_2 population of 80 plants was the largest F_2 population available for the trait and, assuming normal segregation, the size was sufficiently large to differentiate between the expected segregation of a single recessive mutation (expected number of homozygous recessive individuals: 20 ± 4) and the most likely alternative to a single recessive mutation, recessive mutations at two loci (expected number of double recessive individuals in a population of 80: 5 ± 2) with a probability exceeding 99.9%. At the initiation of the F_2 study, the likelihood of the trait being conditioned by three recessive mutations was not considered.

Twenty F_3 kernels harvested from each F_2 plant were evaluated for the HIP phenotype. A total of 80 F_2 plants were evaluated for the HIP phenotype of progeny (F_3) kernels using the HIP assay described above coupled with measurement of absorbance at 820 nm in a Dynamax MRX microplate reader (Dynex Technologies, Chantilly, VA). F_3 kernels were classified relative to the means and standard deviations of the P_i content of 80 kernels of each of the parent genotypes, IDO563 or Js-12-LPA. Individual F_3 kernels were classified as wild-type if the P_i concentration in the kernel was within the interval of two standard deviations above and two standard deviations below the IDO563 mean (0.24 ± 0.14 mg g^{-1}). Individual F_3 kernels were classified as HIP if the P_i concentration in the kernel was within the interval of two standard deviations above and two standard deviations below the Js-12-LPA mean (1.19 ± 0.44 mg g^{-1}). The wild-type and HIP classifications did not overlap. F_3 kernels having P_i concentrations between these two classifications were categorized as intermediate.

F_2 genotype was inferred from $F_{2:3}$ phenotypes by classification into five categories as follows: (i) F_2 plants were classified as homozygous wild-type if no progeny ($F_{2:3}$) kernels were HIP and all $F_{2:3}$ kernels with intermediate P_i concentrations had P_i concentrations below the mid-parent mean. (ii) F_2 plants were classified as heterozygous if at least one progeny ($F_{2:3}$) kernel was HIP and at least one progeny ($F_{2:3}$) kernel was wild-type. (iii) F_2 plants were also classified as heterozygous when HIP kernels were observed, no wild-type $F_{2:3}$ kernels were observed, and kernels of intermediate P_i concentration had concentrations below the mid-parent mean. (iv) F_2 plants were classified as homozygous for the low phytic acid trait if no progeny ($F_{2:3}$) kernels were wild-type and all $F_{2:3}$ kernels with intermediate P_i concentrations had P_i concentrations above the mid-parent mean. (v) F_2 plants were classified as homozygous for the low phytic acid trait if all progeny ($F_{2:3}$) kernels were HIP.

A single F_3 seed of each F_2 plant was planted and advanced in the greenhouse. This process was repeated to produce F_4 plants through single-seed descent. $F_{4:5}$ families were planted in the field at Aberdeen, ID, in 2002. $F_{4:6}$ seed was harvested from those families. Ten individual kernels from each family were evaluated for the HIP phenotype. Total P and the distribution of P as P_i and phytic acid P were determined on whole grain meal of each family as described above. On the basis of our observations of the F_2 families, we assumed that the progeny rows through genetic segregation should fit into three categories: the two parental phenotypes and intermediate phenotypes. Because the distribution of phosphorus as inorganic P, phytic acid P, and other forms is a multivariate distribution we grouped $F_{4:6}$ families into three categories by Euclidean distances using nearest centroid sorting (Anderberg, 1973; SAS Institute, 1997). The clustered procedure used PROC FASTCLUS (SAS Institute, 1997) in SAS using MAXCLUSTERS = 3 with the variables for clustering as P_i, phytic acid P, and other P, all standardized to a percentage of total P.

RESULTS

One M_3 plant from a segregating spike, A95631S-Js-12-333Mu-4-6 (Js-12-LPA), was determined to be homozygous for the HIP phenotype. Another M_3 plant from this same spike, A95631S-Js-12-333Mu-4-8 (Js-12-WT), was determined to be homozygous for the wild-type phenotype. Evaluation of HIP phenotype of five kernels from each plant is shown in Fig. 1A. An additional five kernels were evaluated to confirm the homozygosity of the phenotype. Materials deriving from these individual greenhouse plants are hereafter designated as the genotypes Js-12-LPA and Js-12-WT, respectively.

Observations of the HIP phenotype of greenhouse grown seed of Js-12-LPA were confirmed with seed produced in the irrigated field trials at Aberdeen in 2000. Grain of Js-12-LPA mutant and Js-12-WT had similar total P concentration (5.1 and 5.3 mg g^{-1}, respectively). However, the inorganic P concentration in Js-

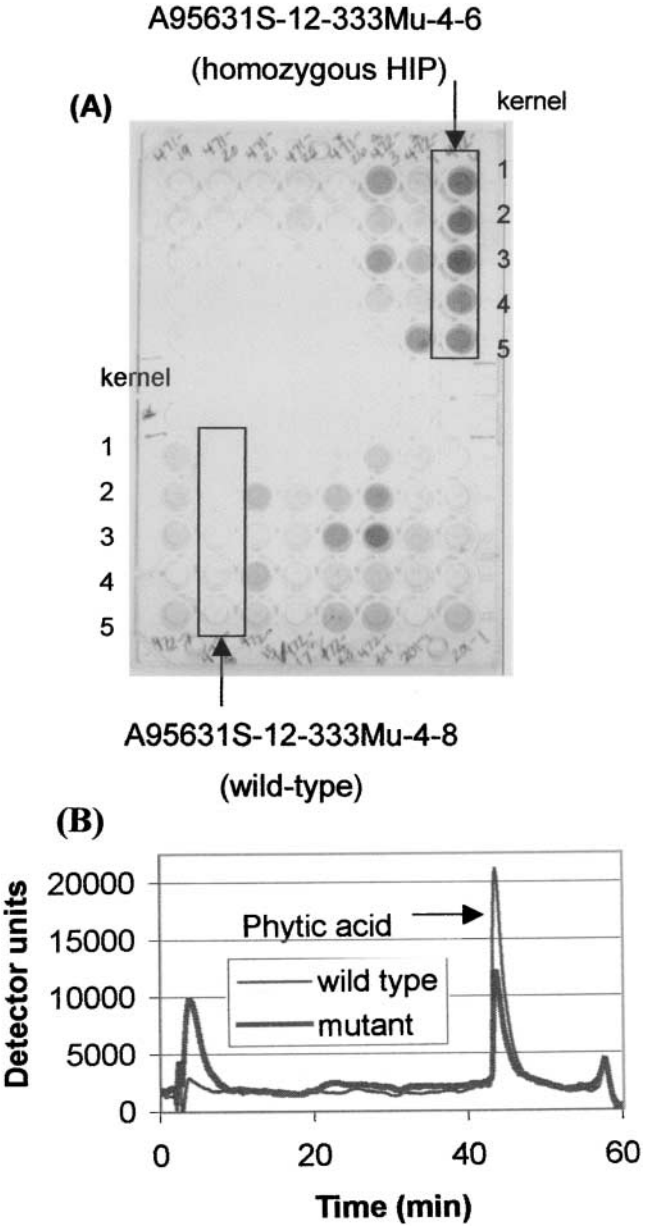

Fig. 1. Js-12-LPA mutant phenotype. (A) Assay for high inorganic phosphate phenotype (HIP). Five kernels per plant were extracted individually and assayed to infer maternal genotype. Wild-type kernels had low inorganic P (low color development). Homozygous mutant kernels had high P_i, producing color. (B) HPLC assay of phytic acid in a single Js-12-WT kernel of wheat and a single Js-12-LPA kernel. Js-12-WT and Js-12-LPA phytic acid P levels were 3.8 and 2.5 mg g^{-1}, respectively. Inositol phosphates of lower phosphorylation were not observed.

12-LPA kernels was five-fold greater than the inorganic P concentration in Js-12-WT kernels (2.6 and 0.5 mg g^{-1}, respectively). Ferric-precipitation and HPLC analyses indicated that the concentration of P present as phytic acid P was reduced from 4.0 mg g^{-1} in Js-12-WT kernels to 2.5 mg g^{-1} in Js-12-LPA kernels, and that unusual accumulations of non-Ins P_6 Ins P's were not observed in Js-12-LPA seed (Fig. 1B).

Milling Analysis

Grain of Js-12-LPA and Js-12-WT produced at Tetonia milled similarly on the Brabender Quadrumat Senior mill (Table 1). Total flour yields were 580 g kg^{-1} for both genotypes. The bran yield was approximately 380 g kg^{-1} for both genotypes. Weight distributions among the flour fractions were essentially identical for Js-12-LPA and Js-12-WT; 27% of flour of both genotypes was recovered in first pass through mill rolls (break flour), and 73% of the flour was recovered in the second pass through the mill rolls (reduction flour).

Total P, phytic acid P, and inorganic P were measured in break flour, reduction flour, shorts, and bran. Total P concentration in the break flour and reduction flour of Js-12-LPA was greater than in break flour and reduction flour of Js-12-WT (Table 1). The concentration of total P in bran of Js-12-LPA, however, was 12% lower than in bran of Js-12-WT. Therefore, it appears that the HIP phenotype alters the distribution of total P within the kernel, increasing the P content of the central endosperm and decreasing the P content of the bran.

The concentration of P as phytic acid in the reduction flour of Js-12-LPA was lower than the concentration of P as phytic acid in reduction flour of Js-12-WT (Table 1). Moreover, concentrations of inorganic P in Js-12-LPA break and reduction flour were nearly four-fold greater than in Js-12-WT break and reduction flour. The concentration of P as phytic acid was 43% lower in bran of Js-12-LPA relative to bran of Js-12-WT.

A relatively low percentage of total P in break and reduction flour was represented by phytic acid P and inorganic P (25 to 43%). While in the bran fractions, 86 to 89% of total P was represented by phytic acid P and inorganic P (Table 1). Lysophospholipids represent 86 to 94% of total starch lipids, and lysophospholipid content can be quantified by multiplying starch P concentration by 16.5 (Morrison, 1964; Morrison et al., 1975; Raeker et al., 1998). Phosphorus content of 12 highly purified soft wheat starches ranged from 0.48 to 0.60 mg g^{-1} dry starch (Raeker et al., 1998). As starch accounts for about 65% of soft wheat flour weight, we can extrapolate that starch-bound P represents between 0.31 and 0.39 mg g^{-1} flour. Therefore, in this study, much of the break and reduction flour P not represented by inorganic P or phytic acid P may be starch-bound lysophospholipid P.

Concentrations of Cd, Co, Cr, Na, Y, and V in all milling fractions were below the analytical limits of detection. Nickel and Pb concentrations in all milling fractions were below the limits of detection, but were above the minimum detection limits in the whole grain sample (Table 2). Concentrations of all minerals were greatest in the bran. Therefore, differences in mineral concentration between the genotypes were most apparent in the bran fraction. The concentrations of only two minerals, Cu and Zn, were significantly affected by the mutant phenotype (Table 2). Js-12-LPA had 21% lower Cu concentration than Js-12-WT in whole grain and 16.5% lower Cu concentration in the bran. Zinc concentration in bran of Js-12-LPA was 33% lower than in bran of Js-12-WT. However, concentrations of Zn in the flour and shorts of the Js-12-LPA and Js-12-WT were similar. The depression in Cu and Zn concentration in bran may be a nutritionally significant detrimental effect of the mutant and will require further characterization in nutrition studies.

Segregation Analysis

Inorganic phosphate analysis of 20 F_3 kernels from each of 80 F_2 plants of the cross Js-12-LPA/IDO563 identified 10 F_2 plants with the homozygous HIP phenotype, 62 F_2 plants with a heterozygous phenotype, and 8 F_2 plants with the homozygous wild-type phenotype (Fig. 2). Chi-square goodness-of-fit values for 1, 2, and 3 gene models with 2 df were 23.2 ($P \leq 0.001$), 8.0 ($P \leq 0.018$), and 102.5 ($P \leq 0.001$), respectively. The observed segregation best fits the expected 1:14:1 ratio of a two-gene trait. However, the observed segregation is not good fit to a two-gene model, perhaps because of imperfect classification of progeny or distorted segregation.

Table 1. Phosphorus concentration in Js-12-WT and Js-12-LPA spring wheat milling fractions from grain produced in Tetonia, ID, 2001.

Genotype and mill fraction	Milling yield	Concentration		
		Total P	Phytic acid P	Inorganic P
	g kg^{-1} grain	mg g^{-1}		
Js-12-WT				
Break flour	157	0.73	0.12	0.05
Reduction flour	427	0.85	0.31	0.06
Shorts	34	4.10	2.90	0.30
Bran	383	8.15	6.70	0.59
Js-12-LPA				
Break flour	159	0.87**	0.08	0.19***
Reduction flour	422	0.91*	0.09*	0.22***
Shorts	35	4.80	1.80*	1.05**
Bran	384	7.15**	3.85***	2.30***

* Mutant is significantly different than the wild-type at the 95% confidence interval.
** Mutant is significantly different than the wild-type at the 99% confidence interval.
*** Mutant is significantly different than the wild-type at the 99.9% confidence interval.

Table 2. Mineral composition of Js-12-WT and Js-12-LPA milling fractions and analysis of variance.

	Al	Ca	Cu	Fe	K	Mg	Mn	Mo	Ni	P	Pb	S	Zn
	μg g^{-1}												
Minimum detection limit	2	2	0.4	2	20	2	0.4	0.4	2	4	2	20	0.4
	First break flour												
Js-12-WT	6.1	150.0	2.35	10.0	895	220	5.90	0.4	–	875		1650	6.65
Js-12-LPA	5.4	160.0	2.15	10.5	885	260	5.30	–	–	970		1600	6.60
Mean	5.7	155.0	2.25	10.3	890	240	5.60	–	–	923		1625	6.63
	Reduction flour												
Js-12-WT	3.2	140.0	3.30	9.0	895	255	7.95	–	–	970		1500	7.35
Js-12-LPA	–	150.0	2.60	8.0	875	255	6.45	–	–	995		1400	6.95
Mean	2.4	145.0	2.95	8.5	885	255	7.20	–	–	983		1450	7.15
	Shorts												
Js-12-WT	9.5	480.0	8.80	30.5	4450	1650	79.00	0.5	–	3900		1750	42.00
Js-12-LPA	10.4	515.0	8.35	25.5	4450	1650	78.50	0.46	–	3650		1750	44.00
Mean	9.9	498.0	8.58	28.0	4450	1650	78.75	0.48	–	3775		1750	43.00
	Bran												
Js-12-WT	14.0	685.0	10.00	49.0	8100	3650	145.00	1.1	–	7800		2550	68.50
Js-12-LPA	13.0	785.0	8.35	33.5	7750	3450	130.00	1.0	–	7000		2500	46.00
Mean	13.5	735.0	9.18	41.3	7925	3550	137.50	1.0	–	7400		2525	57.25
	Whole grain												
Js-12-WT	14.0	400.0	7.25	42.5	4100	1650	73.50	0.6	9.1	3850	13.5	2000	36.00
Js-12-LPA	8.0	435.0	5.70	27.5	3800	1650	59.50	0.9	8.2	3550	7.6	1950	30.50
Mean	11.0	4185.0	6.48	35.0	3950	1650	66.50	0.8	8.6	3700	10.6	1975	33.25
SE†	0.9	14.3	0.18	8.0	194	89	3.70	0.1	–	146	–	50	3.50
	ANOVA *F* value												
Genotype (G)	ns	ns	8281**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Mill Fraction (M)	35.8***	605***	596***	6.78**	456***	463***	453***	ns	–	661***	–	138***	81.2***
G × M	ns	ns	6.4**	ns	ns	ns	ns	ns	–	ns	–	ns	4.1*

* Significant at $P = 0.05$.
** Significant at $P = 0.01$.
*** Significant at $P = 0.001$.
† Standard error for each genotype within each flour or grain fraction.

In $F_{4:6}$ seed, the inorganic P concentration ranged from 0.16 to 2.44 mg g^{-1} or 5 to 61% of total P. Phytic acid P concentration ranged from 1.37 to 4.89 mg g^{-1}; while total P ranged from 2.77 to 6.82 mg g^{-1}. Values for the parents grown within the field trial were: Js-12-LPA had 1.88 mg g^{-1} inorganic P or 43% of total P (3.87 mg g^{-1}), and IDO563 had 0.23 mg g^{-1} inorganic P or 6% of total P (4.41 mg g^{-1}). The distributions of families based on inorganic P as a percentage of total P and phytic acid P as a percentage of total P were continuous rather than discrete, without clear genotypic classes (Fig. 3).

By the cluster analysis, 15 of the $F_{4:6}$ families clustered into a low inorganic P group/high phytic acid P (cluster average: 11% of P as inorganic P and 87% of P as phytic acid), 53 families into an intermediate group (cluster average: 13% of P as inorganic P and 64% of P as phytic acid P), and 13 families into a high inorganic P/low phytic acid P group (cluster average: 42% of P as inorganic P and 40% of P as phytic acid P). The expectation for a two-gene model in a population size of 81 for

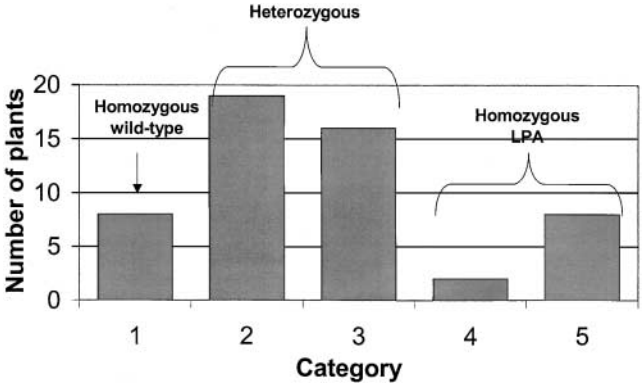

Fig. 2. Distribution of genotypes assigned to F_2 plants from the cross Js-12-LPA/IDO563. F_2 plants were classified into five categories: (1) Homozygous wild-type if no progeny ($F_{2:3}$) kernels were HIP (high inorganic phosphate) and all $F_{2:3}$ kernels with intermediate P_i concentrations had P_i concentrations below the mid-parent mean; (2) Heterozygous if at least one $F_{2:3}$ kernel was HIP and at least one $F_{2:3}$ kernel was wild-type; (3) Heterozygous if HIP kernels were observed, no wild-type $F_{2:3}$ kernels were observed, and kernels of intermediate P_i concentration had concentrations below the mid-parent mean; (4) Homozygous for the low phytic acid trait if no $F_{2:3}$ kernels were wild-type and all $F_{2:3}$ kernels with intermediate P_i concentrations had P_i concentrations above the mid-parent mean; (5) Homozygous for the low phytic acid trait if all $F_{2:3}$ kernels were HIP.

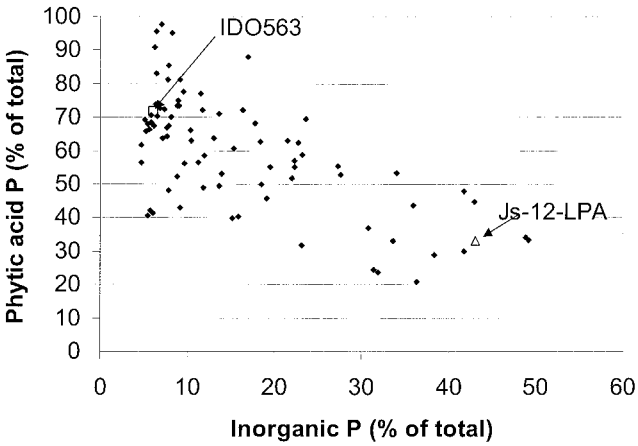

Fig. 3. Distribution of $F_{4:6}$ families for inorganic P as a percentage of total P and phytic acid P as a percentage of total P.

inheritance of the HIP phenotype is that 15.5 families would be found for each of the parental classes and 50 individuals would be found in intermediate classes. The three cluster distribution of 15:53:13 fits a two-gene model of the 81 families (15:51:15) very well ($\chi^2 = 0.60$, 2 df, $P \leq 0.74$) and does not fit the one gene model of 35:11:35 for the 81 families ($\chi^2 = 208$, 2 df, $P \leq 0.001$).

The mean kernel weight of families from the three clusters did not differ significantly. The mean kernel weight of families from the high inorganic P/low phytic acid P cluster was 37.1 mg kernel^{-1}, while the mean kernel weight of families from the low inorganic P group/high phytic acid P cluster was 35.1 mg kernel^{-1}, and the mean kernel weight of families from the large intermediate cluster was 34.7 mg kernel^{-1}. This suggests that the mutations conferring the low phytic acid trait do not affect general grain development but are specific to P storage.

DISCUSSION

The levels of inorganic P measured in Js-12-LPA and Js-12-WT wheat from the initial Aberdeen field production are within typical ranges for low phytic acid and wild-type cereals (Raboy, 2002). However, the overall reduction in phytic acid P (37%) in Js-12-LPA was lower than that observed in barley and maize mutants (≈50 to 95%). This may reflect a greater buffering capacity conferred by the hexaploid wheat genome relative to the diploid barley and maize genomes. Accumulations of lower inositol polyphosphates were not observed in Js-12-LPA, indicating that this mutant is not of the *lpa2*-type.

Flour extractions of both Js-12-LPA and Js-12-WT wheat produced at Tetonia were relatively low. But in our experience, these extractions are typical of late-planted grain produced at Tetonia. The 2001 Tetonia crop was planted unusually late because of the late timing of the harvest of the Southern California increase. Therefore, our initial conclusion is that there are not obvious adverse effects of the HIP phenotype on milling.

Phytic acid was primarily sequestered in the bran of both Js-12-LPA and Js-12-WT wheat. However, the HIP phenotype altered the distribution and compositional profile of P within the kernel. Phytic acid P concentration was reduced 43% in bran of Js-12-LPA, while inorganic P concentration was increased three- to four-fold in all milling fractions of Js-12-LPA relative to Js-12-WT. Phytic acid becomes a component of animal feed through bran. Nonruminant animals do not utilize phytic acid P, which is primarily excreted and becomes an important environmental concern. Therefore, the Js-12-LPA trait may have a positive impact on the nutritional quality of the wheat bran fed to animals and may reduce the environmental impact of animal waste P.

Phytic acid chelates divalent cations, which are principally sequestered in the bran. It is possible that the decreased concentration of Cu and Zn in bran of Js-12-LPA was a consequence of the decrease in phytic acid concentration. To date, no other *lpa* mutation has been found to reproducibly impact seed or seed fraction mineral content. These minerals did not appear to be present in higher concentrations in other milling fractions of Js-12-LPA. Further studies are underway in our laboratory to evaluate the relationship between the *lpa* trait and mineral content in wheat.

The mutagenesis of Js-12 produced a heritable low phytic acid phenotype. The distribution of phosphorus as inorganic P relative to phytic acid P is a quantitative trait affected by seed characteristics. This limits the resolution of our segregation data. The simplest explanation of mutagenesis for a new phenotype is the assumption of perturbation of a single gene. Mapping of low phytic acid mutants of rice, barley, and maize has indicated that these are single-gene perturbations (Larson et al., 1998, 2000; Raboy et al., 2001). However, our inheritance data of F_2 and $F_{4:6}$ families is inconsistent with the assumption of a single locus model and suggests the involvement of multiple loci in Js-12-LPA. The observed frequencies of parental phenotypes are most consistent with a two-gene model. Mapping studies with Js-12-LPA are needed to confirm this hypothesis. Other possible explanations for the observed data in the F_2 population include a naturally occurring mutation in the IDO563 parent and a mutation with partial or 'leaky' expression. The first alternative seems unlikely because IDO563 is a first backcross sib of the donor germplasm used for mutagenesis and should be similar to the donor line, given that very limited variation has been reported in wheat or other agronomic crops in the absence of induced mutations (Raboy, 2002). The second alternative also seemed unlikely given the phenotypes observed in the RI lines. A leaky mutation should produce many lines with variable expression for the HIP phenotype. Instead, we observed that most of the genotypes were internally consistent. The number of RI lines with variation in HIP similar to the total range observed for the cross was small enough in number to be consistent with the expected number of heterozygotes in a two-gene model.

The low phytic acid trait is heritable and was recovered with sufficient frequency to be used in breeding. Backcross breeding using the HIP phenotype has successfully moved the Js-12-LPA phenotype into advanced spring wheat breeding materials (Souza and Guttieri, 2003, unpublished data).

The full HIP phenotype requires further investigation. Ideally, the suppression of phytic acid should have minimal pleiotropic effects on the normal functionality of the wheat kernel within the milling and baking industry. Moreover, the HIP phenotype ideally should be agronomically neutral. The Js-12-LPA wheat characterized in this study is agronomically unacceptable because of reduced stature, markedly weak straw, and dramatically reduced grain yield. Genotypes derived from mutagenesis often have undesirable traits that will segregate independently of the desired trait. Studies to address the agronomic and end-use quality effects of the HIP phenotype currently are underway in our laboratory.

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